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The objectives of the present study were to determine the mechanisms of ion transport across mineralizing epithelia of barnacles, in particular to quantify the effects of altered protein structure on crystal form and arrangement and the effects of the molting hormone 20-OH ecdysone on calcium carbonate deposition rate and growth increment formation. The main technical focus was to develop new methods for monitoring and measuring the mineralization process. The studies of biomineralization by the principal investigator undertaken in the early 1950s demonstrated for the first time that $^{45}Ca$ could be used to measure the rate of mineral deposition in shells of marine molluscs. These studies conducted during 1992-1994 demonstrate, for the first time, that elemental probes, such as strontium and manganese, can substitute for calcium in mineral formation. Semi-quantitative electron probe x-ray images of Sr and Mn revealed that their incorporation is cyclical, directly related to addition of growth increments, and proportional to the amount of element in the sea water environment. This use of elemental probes, coupled with the development of a functional in vitro mineralizing epithelial preparation, makes possible a new approach to invertebrate mineralization studies.



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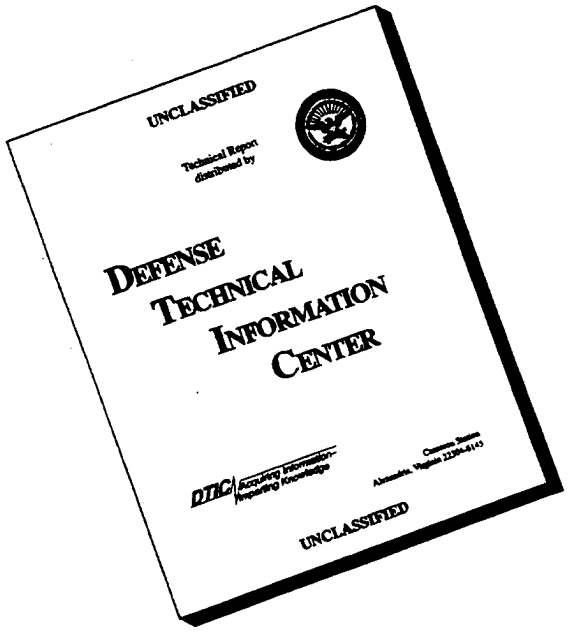


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ANNUAL PROGRESS REPORT

Grant #: N00014-92-J-1211 R&T Code: 4412114

PRINCIPAL INVESTIGATOR: Karl M. Wilbur, Ph.D. (died, 9/94)
Ann LeFurgey, Ph.D., Acting PI

INSTITUTION: Duke University
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GRANT TITLE: Calcium Carbonate Mineralization

REPORTING PERIOD: 15 April, 1994 - 30 September, 1994 and Final for entire award period

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OBJECTIVES: To investigate (1) the effects of altered protein matrix on mineral microstructure of barnacle shell and molluscan shell; (2) the effects of the molting hormone 20-OH ecdysone on barnacle growth rate; (3) the feasibility of devising an in vitro culture system of barnacle shell plus mineralizing epithelium separated from the influences of the intact organism; (4) the elemental composition and structure of mineralizing and cuticle- or matrix-forming cells in the cyprid and juvenile barnacle by electron probe x-ray microanalysis.

APPROACH: **Culture of barnacles on coverslips** for (1) measurement of growth rates by scanning electron microscopy; (2) measurement of growth rates by sequential determinations of buoyant weights of barnacles and determination of the effects of 20-OH ecdysone on growth; (3) observation of cyprid carapace and juvenile and adult shell microstructure and composition by scanning electron microscopy and energy dispersive x-ray analysis. **Development of a simple barnacle *in vitro* system** for study of the mechanisms of $CaCO_3$ deposition.

ACCOMPLISHMENTS:

**1992-1993.** A method for growth measurements of living barnacles, in terms of fresh weight, dry weight, shell weight, and organic weight, was developed to measure daily growth rates and the effects of hormones such 20-OH ecdysone and a protein glycosylation inhibitor, tunicamycin (TM), on growth. The molting hormone is without significant effect on growth rate; however, TM reduces the rate of calcium deposition in B. amphitrite.

**1993-1994.** Barnacles growing in artificial sea water, to which various marker elements are added (1 mM or less), can

incorporate Sr and Mn but not Ba into the shell and/or organic matrix. Pb at trace levels inhibits formation of the calcium carbonate shell, but is not itself incorporated into the shell. For Sr and Mn, the amount incorporated into the shell is directly proportional to the amount of the element in the sea water environment.

**SIGNIFICANCE:** The shell-epithelial *in vitro* system provides a simple method for investigating the mechanisms by which an epithelium transports ions into a microspace and brings about $CaCO_3$ deposition; The fact that Sr or Mn can be built into a $CaCO_3$ shell suggests the shell organic matrix will provide a substrate for more than the primary mineral calcium. Incorporation of such elemental probes as Sr and Mn provides an experimental means to track the course of movement of these calcium substitutes through the cells of the mineralizing epithelium, the extrapallial space, and the shell, in the intact barnacle as well as the shell-epithelial *in vitro* system.

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**Since 15 April, 1994:**

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Hockett, D.N.C., P. Ingram, D.A. Kopf, and A. LeFurgey. Barnacle shells analyzed by x-ray imaging can provide a chronological record of metal contamination in estuaries.

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# THE TIME COURSE OF CALCIUM DEPOSITION IN SHELLS OF THE BARNACLE (*BALANUS AMPHITRITE AMPHITRITE*) DURING CYPRID-JUVENILE METAMORPHOSIS

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The morphology of balanomorph barnacles during metamorphosis from the cyprid larval stage to the juvenile has been examined by light microscopy and scanning electron microscopy (SEM).[1,2] The free-swimming cyprid attaches to a substrate, rotates 90° in the vertical plane, molts, and assumes the adult shape. The resulting metamorph is clad in soft cuticle and has an adult-like appearance with a mantle cavity, thorax with cirri, and incipient shell plates. At some time during the development from cyprid to juvenile, the barnacle begins to mineralize its shell, but it is not known whether calcification occurs before, during, or after ecdysis. To examine this issue, electron probe x-ray microanalysis (EPXMA) was used to detect calcium in cyprids and juveniles at various times during metamorphosis.

Laboratory-raised, free-swimming cyprid larvae were allowed to settle on plastic coverslips in culture dishes of seawater. The cyprids were observed with a dissecting microscope, cryopreserved in liquid nitrogen-cooled liquid propane at various times (0-24 h) during metamorphosis, freeze dried, rotary carbon-coated, and examined with scanning electron microscopy (SEM). EPXMA dot maps were obtained in parallel for qualitative assessment of calcium and other elements in the carapace, wall, and opercular plates.[3]

Calcium was not detected in the carapace of cyprids, regardless of whether they were free-swimming or in the early stages of attachment to the substrate prior to metamorphosis (Fig 1A, B). During ecdysis, calcium was not present in the carapace, but was detected in the underlying incipient shell plates (Fig. 2A, B). Immediately after ecdysis, calcium was detected in the opercular plates, but not in the wall plates (Fig. 3A, B). Within 30 min of transformation, calcium was detected in both the opercular and wall plates (Fig. 4A, B); the opercular plates appeared to have more calcium than the wall plates. Thus, it appears that calcium deposition begins either just prior to or during ecdysis and that calcium deposition occurs in the opercular plates before the wall plates.[4]

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4. This work was supported by the Office of Naval Research #N00014-92-J-1211, NIHS10RR06692, and NSF DIR9106607. The authors thank Dr. Dan Rittschof of the Duke University Marine Laboratory for providing cyprid larvae.

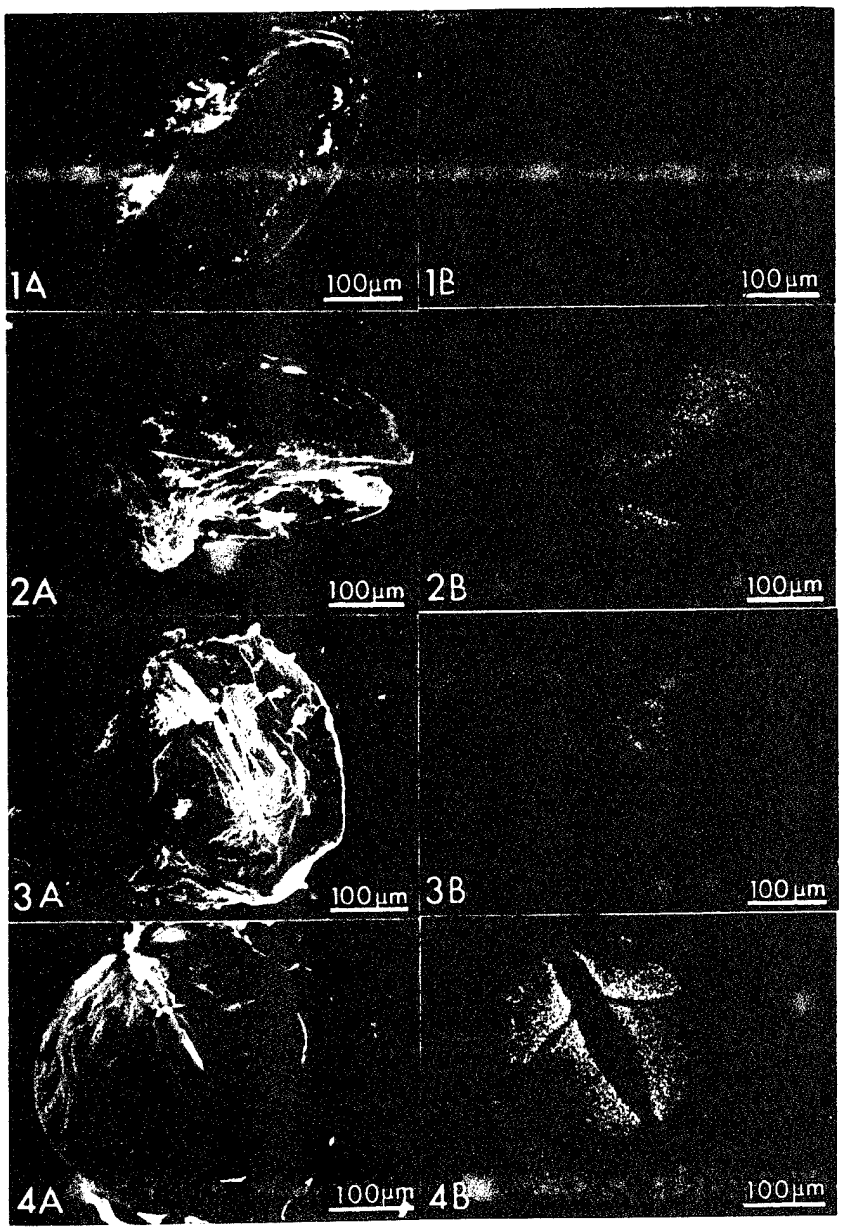


FIG. 1-SEM (A) and calcium EPXMA dot map (B) of free-swimming cyprid.
FIG. 2-SEM (A) and calcium EPXMA dot map (B) of attached cyprid during ecdysis
FIG. 3-SEM (A) and calcium EPXMA dot map (B) of juvenile immediately after ecdysis.
FIG. 4-SEM (A) and calcium EPXMA dot map (B) of juvenile 30 min after ecdysis.

# MICROSCOPY AND MICROANALYSIS OF CALCIFICATION IN THE DEVELOPING AND ADULT BARNACLE

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The barnacle begins its development in its first larval stage, the naupilus, and matures to the larval cyprid stage. Permanent attachment of the barnacle occurs between the cyprid and juvenile stages. Soon after, calcification takes place and the shells of the barnacle grow upward. The application of secondary electron imaging (SEI) and electron probe microanalysis (EPXMA) has defined the time course of calcification and shell growth in the barnacle.[1] Freeze-dried cryosections of the larval cyprid stage can also be employed for localization of calcium at the cellular level with scanning transmission electron microscopy (STEM)/EPXMA; however these sections are unstained, thereby offering very little contrast and making the recognition of ultrastuctural compartments difficult. In an attempt to identify these structures, conventional fixation staining and transmission electron microscopy (TEM) will be used. The light microscopic study of the cyprid has been extensive,[2] but very little ultrastructural research has been conducted. The goal of this study is twofold: to use light microscopy (LM) and TEM as guides in identifying structures of the STEM/EPXMA cryosections; and to follow the growth of the barnacle by using STEM/EPXMA to detect calcium tracers in the shell.

After fixation in 2% buffered glutaraldehyde and embedding in epoxy, the cyprid larvae were cut into semi-thick (350 nm) and thin (70nm) sections. To observe the three-dimensional structure of cells and subcellular regions, a computer reconstruction was created from one hundred and eight serial semi-thick longitudinal sections that were collected and digitized (Fig.1). An additional computer reconstruction using the cross-section of the cyprid is currently in progress. Characteristic body regions including the oil cells, appendages, and compound eye were identified in the light microscopic and TEM images. An enlarged montage of a longitudinal cyprid section was assembled from the TEM images (Fig.2). From this heterogeneous cell population, cell substructures have been identified including nuclei, mitochondria, muscle fibers, etc.

EPXMA is useful to further understand biomineralization and growth in the cyprid. EPXMA cannot distinguish between isotopes of calcium; therefore, several calcium substitutes have been tested as tracers of calcium.[3] Manganese and strontium have been successfully incorporated into the barnacle shell and detected by EPXMA, proving to be useful calcium tracers (Fig.3). We are exploring the possibilities of using barium as an additional tool in tracing calcium movement, but previous studies have shown that Ba at micromolar concentrations induces structural deformation.[4]

We are still working to identify the ultrastructure of the cyprid larvae and to understand the methods of biomineralization in the barnacle. The recognition of cellular organelles in the TEM image allows us to refer to the correlating location on the unstained cryosection and grasp the relative distribution levels of calcium given by the EPXMA image. The inclusion of metals into the shell depicts the flow of calcium as the barnacle undergoes calcification as a means of growth. This combined approach employing TEM, STEM and EPXMA as well as using SEI and EPXMA to detect calcium tracers will allow for better comprehension of the mechanics of biomineralization and growth of cyprids and adult barnacles.

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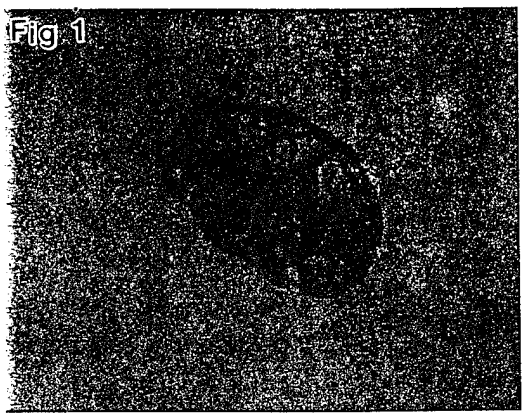
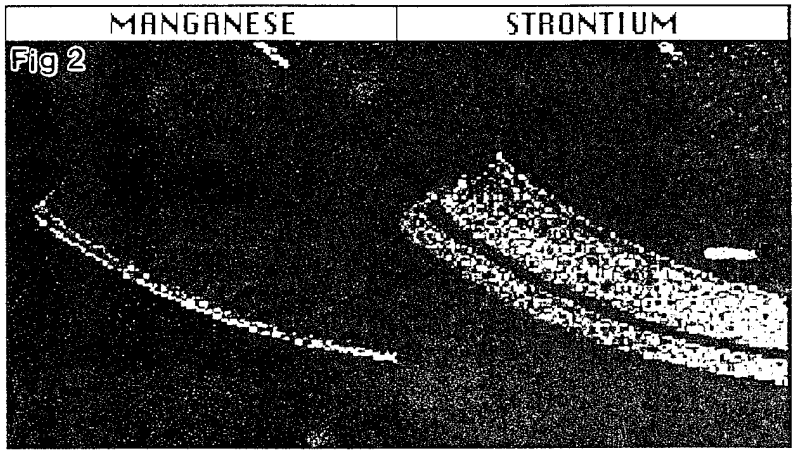
5. This work supported by the National Science Foundation (NSF BIR9106607) and the Office of Naval Research (N00014-92-J-1211)
The authors extend special thanks to Dr. Peter Ingram for his assistance.





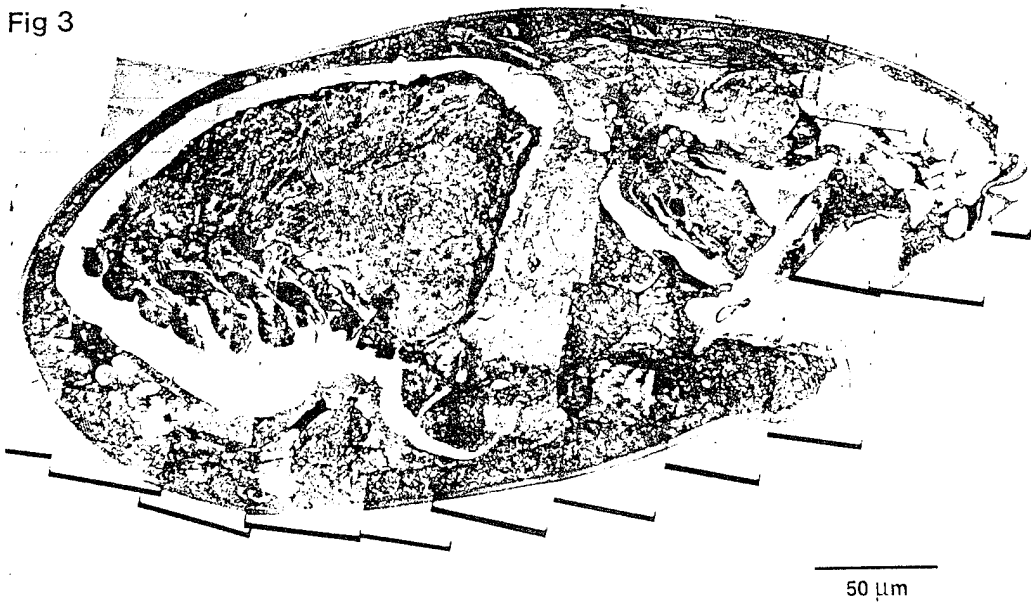




Figure 1- A digitized image taken from 3-D computer reconstruction of the cyprid.
Figure 2- An enlarged montage of a longitudinal cyprid section assembled from TEM images.
Figure 3- EPXMA map of a barnacle shell pulsed with Mn and Sr. The bands show detection of Mn and Sr respectively.

3703

CYTOMEGALOVIRUS MODULATES THE $CA^{2+}$-RESPONSE TO VASOPRESSIN AND ATP IN FIBROBLAST CULTURES

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The free $Ca^{2+}$ concentrations in nucleus ($[Ca^{2+}]_n$) and in cytosol ($[Ca^{2+}]_c$) of cultured human embryonic lung (HEL) fibroblasts were estimated by confocal laser microscopy using the $Ca^{2+}$-indicator Indo-1. In resting HEL cells the free $[Ca^{2+}]$ significantly increased upon human cytomegalovirus (HCMV) infection. The ratio between $[Ca^{2+}]_n$ and $[Ca^{2+}]_c$ was not affected. Following stimulation by ATP or [$Arg^8$] vasopressin (AVP), a differential $Ca^{2+}$-response of the HCMV-infected HEL cells was observed. While uninfected cells were highly sensitive to AVP and only poorly sensitive to ATP, infected cells showed a high responsiveness to ATP but not AVP. This switch in sensitivity to the agonists was first observed at 24 hr post infection. The $Ca^{2+}$-rise following ATP or AVP stimulation was derived from intracellular $Ca^{2+}$ stores. The magnitude of the ATP-induced $Ca^{2+}$-rise increased upon infection. In contrast to non-infected cells where $[Ca^{2+}]_n > [Ca^{2+}]_c$, no nucleo-cytosolic $Ca^{2+}$-gradient was observed in infected cells. It is concluded that HCMV infection significantly interferes with $Ca^{2+}$ homeostasis in HEL cells which could be related to the pathogenesis of the disease.

3704

CONTROL OF CELL Ca PERMEABILITY DURING STIMULATION OF THE GASTRIC PARIETAL CELL.

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Cholinergic agents produce a transitory $Ca^{2+}$ release from internal stores, followed by sustained $Ca^{2+}$ entry from the extracellular medium. The mechanisms that control $Ca^{2+}$ entry during stimulation are not yet defined. We have studied the relationship between $Ca^{2+}$ release from internal stores, $[Ca^{2+}]i$ and $Ca^{2+}$ permeability in rabbit parietal cells preloaded with fura-2. Carbachol (Cchol) induced a dose-dependent effect on $Ca^{2+}$ release and $Ca^{2+}$ permeability. Inhibition of ER $Ca^{2+}$ ATPase by thapsigargin (3μM,THG) slightly increased $[Ca^{2+}]i$ with a large increase in $Ca^{2+}$ permeability. Sodium nitroprusside (SNP), a nitric-oxide donor, appeared not to have an effect on membrane permeability. Clamping $[Ca^{2+}]i$ with BAPTA buffered changes in $[Ca^{2+}]i$ induced by Cchol, THG and $Ca^{2+}$ pulses without affecting the increases in $Ca^{2+}$ permeability. SNP in these conditions increased $Ca^{2+}$ permeability. The results suggest that the increase in $Ca^{2+}$ permeability: i) is not secondary to an increase of $[Ca^{2+}]i$, ii) is linked to the emptying of $Ca^{2+}$ reservoirs, and iii) may be related to the metabolism of nitric-oxide. The participation of receptor operated channel cannot be ruled out.

FASEB JOURNAL 9: A639, 1995

3705

EFFECT OF CHRONIC FLUORIDE EXPOSURE ON CALCIUM TRANSPORT PROTEINS IN RAT KIDNEY.

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Chronic exposure to high levels of fluoride (F) has effects on several calcium-dependent processes. An effect of chronic F administration on calcium (Ca) transport protein expression has not been reported. In this study, three groups of female SD rats (n=10/group) were given NaF by ip injection (0, 2.5, and 5.0 mg F/kg/day) for six weeks. Twenty four-hour Ca and F balance studies were conducted each week. At the end of the study, the rats were anesthetized with pentobarbital and renal clearances of inulin (GFR) and Ca were determined. Plasma was analyzed for PTH. Kidney tissues were harvested for immunochemical studies with specific antibodies to the plasma membrane Ca-pump (PMCA), 28kDa and 9kDa calbindin proteins. The results showed no significant F-attributable difference in plasma PTH or Ca balance between the control and F-injected groups. The clearance studies showed no difference in GFR, Ca clearance, or fractional Ca clearance (tubule-specific transport, however, was not measured). A significant elevation of plasma Ca was seen in the 5.0 mg F/kg treated group relative to controls ($p=0.02$). Quantitation of protein antigen in each group also showed a significantly higher amount of PMCA in kidney tissue from rats receiving daily injections of 5.0 mg F/ kg ($p=0.013$), when compared to controls. No significant differences in the amounts of 28kDa or 9kDa calbindin antigens were seen among the groups. These results suggest that chronic exposure to high doses of NaF may alter Ca resorption in the kidney by an effect on PMCA turnover or expression. This work supported in part by MCG Research Institute & NIDR DE06113.

3706

THE ONSET OF BIOMINERALIZATION DURING CYPRID TO JUVENILE METAMORPHOSIS OF THE BARNACLE (Balanus amphitrite amphitrite)

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During the final metamorphosis of the barnacle, the free-swimming cyprid larva attaches to a substrate, molts, and assumes the adult form. At some time during this transformation, the barnacle starts to mineralize its shell; however, it is not known whether this calcification occurs before, during, or after molting. To address this issue, we observed laboratory-raised, free-swimming cyprid larvae during metamorphosis by using light and electron microscopic techniques including time-lapse movies and electron probe X-ray microanalysis (EPXMA) imaging. In the free-swimming cyprid larva, Ca was not present in the carapace, but was detected in the underlying body as revealed by EPXMA; no polarized light signal was detected in the larva. During ecdysis, Ca was not detected in the carapace, but was present in the underlying incipient shell plates. Immediately after molting, Ca was present in the opercular plates, but not in the wall plates. Thirty minutes after molting, Ca was detected in both the opercular and wall plates and a polarized light signal was observed. After 24 h, Ca was present in all plates and polarized light signals were detected. These observations suggest that Ca deposition begins just prior to or during ecdysis and that the shell becomes ordered within the first 30 min after transformation. Supported by ONR (N00014-92-J-1211, N00014-94-1-0818), NIHS10RR06692, NSF DIR9106607, and the Duke Univ. Marine Biomedical Ctr.

## MUSCLE AND LOCOMOTOR ADAPTATION; PHYSIOLOGICAL ECOLOGY; TEMPERATURE ADAPTATION AND ENERGETICS; RESPIRATION AND ACID/BASE (3707-3708)

3707

CHANGES IN MUSCLE MASS AND OXIDATIVE CAPACITY AFTER 10 WEEKS DETRAINING IN GOLDEN MANTLED GROUND SQUIRRELS.

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A previous study (Wickler et al *AJP* 261: R1214, 1991) indicated that hibernating ground squirrels demonstrated muscle atrophy but an increase in the oxidative capacity (as measured by citrate synthase). This study did not examine changes in muscle that may have occurred between capture and hibernation. The present study measured changes in skeletal muscle that occurred after 10 weeks of captivity in a laboratory setting prior to hibernation. 19 animals were trapped between July and August (1994) in the Sierra Nevada Mountains. The control group (n=11) was sacrificed immediately, while the experimental group (n=8) was caged (detrained) for ten weeks in an animal facility (food and water ad libitum and room temperature, 22°C). There was significant atrophy in the gastrocnemius/plantaris of experimental animals ($0.93 \pm 0.16$ vs. $0.82 \pm 0.15$ g) as indicated by ANCOVA with carcass mass as a covariant In addition, experimental animals had significant increases in oxidative capacity expressed either mass-specific ($30.1 \pm 4.20$ vs. $53.7 \pm 6.57$ μmoles/g/min ) or as total ($28.0 \pm 5.9$ vs. $44.1 \pm 10.8$ μmoles/min ). These results are consistent with an earlier study (Tseng et al. *FASEB J.* 8:A3324, 1994), and demonstrate that Golden-Mantled Ground Squirrels undergo muscle atrophy and an increase in oxidative capacity prior to hibernation. [Supported by an NIH grant, RAP 1 R15 AR-39893-01A2 to DFH & SJW]

3708

EFFECT OF 90 DAYS DETRAINING ON MUSCLE MASS AND OXIDATIVE CAPACITY IN DESERT IGUANAS, (*Dipsosaurus dorsalis*).

Christine S.I. Tseng*, Steven K. Teh*, Charles Yeloushan*, Donald F. Hoyt and Steven J. Wickler, California State Polytechnic University, Pomona, CA 91768

Gleeson (*J Comp Physiol* 129:123, 1981) reported that lizard muscle is less responsive to activity training than mammalian muscle. The question we posed was how responsive is reptilian muscle to detraining? Iguanas were captured and sampled immediately (n=13) or placed into cages (n=14) with food and water ad libitum for 90 days. Two hindlimb muscles were sampled: The iliofibularis (IF) and the gastrocnemius/soleus. Carcass masses (body mass minus viscera) were not different between control and experimentals (58.0±4.0 vs 64.6±3.7 g, respectively) but muscles atrophied. The gastrocnemius/soleus decreased from 11.8±0.2 to 8.7±0.6 mg/carcass mass. The IF decreased from 2.72±0.05 to 2.34±0.09 mg/g carcass mass. Citrate synthase activity, an indicator of oxidative capacity was not different when expressed per g muscle for the gastrocnemius/soleus (4.12±0.32 vs 4.63±0.28 U/g for controls and experimentals, respectively) or for the IF (4.71±.30 vs 5.03±0.22 U/g for controls and experimentals, respectively). [Supported by an NIH grant, RAP 1 R15 AR-39893-01A2 to DFH & SJW].

# BARNACLE SHELLS ANALYZED BY X-RAY IMAGING CAN PROVIDE A CHRONOLOGICAL RECORD OF METAL CONTAMINATION IN ESTUARIES

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Due to the rapid partitioning of toxic metals into estuarine sediments, simple water sample analysis might fail to detect the pollutant. Barnacle shells may provide a unique indicator of estuarine metal pollution events. Barnacles produce new bands of calcium carbonate shell at every tidal inundation.[1] If metals present in the water are incorporated into the shell and can be detected, then a sequential record of water quality could be created by imaging the metal content in the shells by electron probe x-ray microanalysis (EPXMA). Furthermore, since the geochemical cycling of many earth elements, (e.g. strontium,) is increased by global warming or acid precipitation, samples of barnacle shells collected now could be compared to historical samples to assess changes in the environment.[2, 3] The present study evaluated the potential of a barnacle bioindicator of metal pollution in a controlled laboratory study.

Barnacles (*Balanus amphitrite amphitrite*) in the cyprid larval stage were placed on plastic cover slips for two days to allow for settling and attachment. The cover slips containing the attached juvenile barnacles were then placed into artificial sea water (ASW) at 20°C and fed algae for one week. After one week, the barnacle diet consisted of brine shrimp (*Artemia sp.*). Barnacles were then exposed to ASW dosed with various metals. The barnacles were placed in the metal enriched ASW for varying times (4 hours to two days) and then placed back into normal sea water so that the newly deposited shell bands would grow off the substrate. Controls included dosed abiotic shells and live organisms placed in normal ASW. A dose response experiment was also performed where various levels of Sr enriched ASW (from 0.1 mM to 1mM) were alternated with manganese enriched ASW. These enrichment levels were well within natural concentrations of Sr in sea water which can range up to 0.9 mM.[4] Manufacturer specification of the artificial sea water used in these experiments (Instant Ocean) state that Sr is present at 0.1 mM. After treatment, barnacles were removed from their shells and shells were separated by soaking for about 15 minutes in a 5.25% sodium hypochlorite solution. The shells were then mounted on carbon stubs and rotary carbon coated in preparation for SEM analysis and EPXMA digital mapping. The characteristic x-ray peaks were identified and peak counts quantified by top hat filtering and multiple least squares fitting to a reference set. The ratio between the metal and the calcium was used to assess the metal level in order to minimize variations in specimen-detector geometry and in sample thickness.

Manganese and lead were incorporated into the shells; however, Pb drastically altered the level of Ca in the shells, invalidating the use of the ratio method in this case (Figure 1). Strontium was also incorporated in a dose dependent fashion (Figures 2 and 3). The presence of Sr and Mn presumably attenuated somewhat the Ca peak due to Ca x-ray absorption by these heavier elements. However, in adjacent areas of the same shell, one area containing Sr and one area not, the maximum change in the magnitude of the Ca peak was 8%. Abiotic controls had the same Sr:Ca ratio as untreated shells suggesting, at least for Sr, that adsorption processes were minimal. Crustaceans are highly sensitive to copper and zinc and doses as low as 10 μM were lethal. Neither uranium (hexavalent) nor nickel were detected when administered at the highest non-lethal doses (1 mM for U and 10 μM for Ni).

There are at least three possible explanations for metal incorporation into barnacle shells. First, metals which are calcium analogs could be transported via calcium channels and undergo a similar biomineralization process as calcium carbonate formation.[5] Second, the metal could reach the extra-pallial space through either transcellular routes or directly from the external water via pores in the shells. The extra-pallial space is at a higher pH due to active proton exchange[5] and at increasing pH, metals are more likely to precipitate as either carbonates or hydroxides.[6] A third possibility is that the metals adsorb to the organic matrix of the shell soon after the matrix is produced, but before calcium carbonate is deposited over the matrix.

Barnacle shell plates can reflect the level of at least some metals present in sea water. More sensitive microprobe techniques (e.g. SIMS or LAMMA) may prove useful in detecting lower levels of metals such as nickel and could prove to be ideal in the development of this bioindicator of estuarine metal pollution.[7]

References

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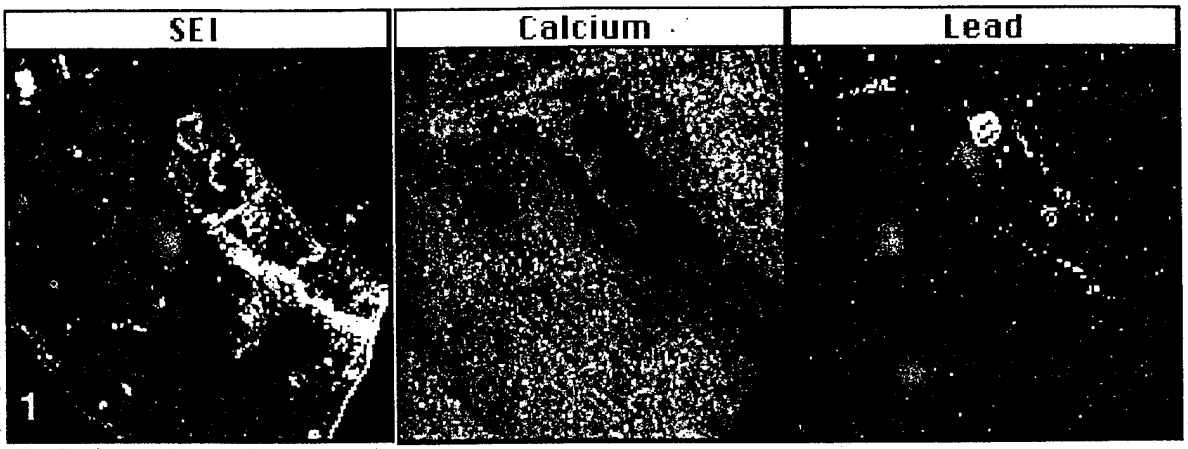


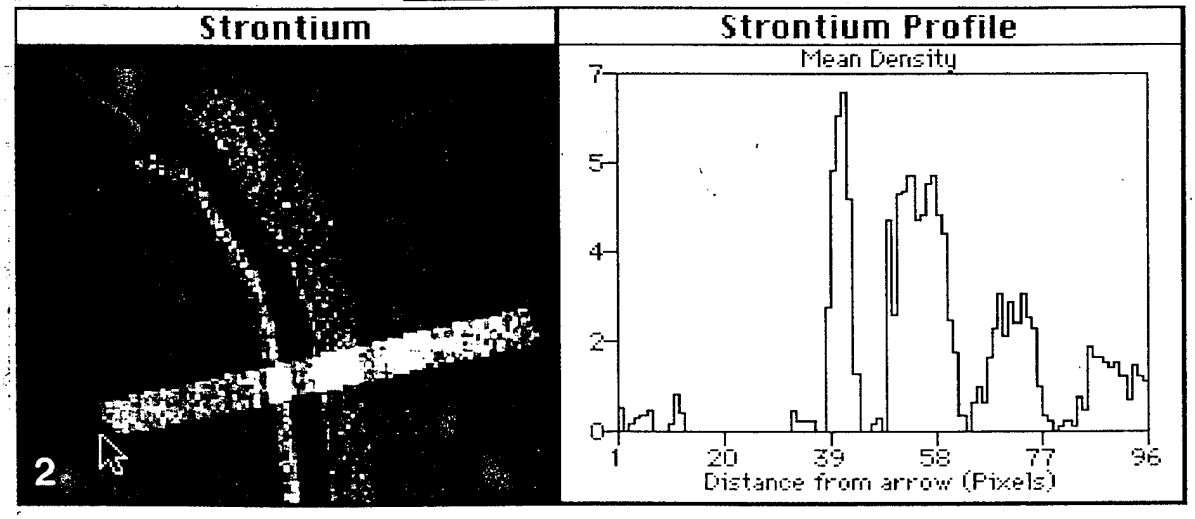


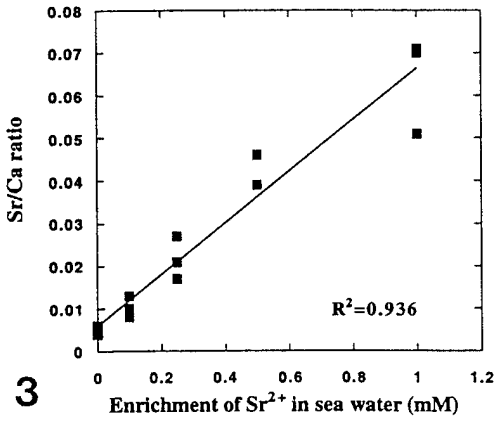


FIG. 1. Digital lead and calcium x-ray images (35x) of barnacle lateral wall plate showing the large reduction in shell calcium in areas corresponding to growth in lead enriched ASW.
FIG. 2. Digital strontium x-ray image (50x) of the scutum shell plate of a barnacle exposed to 4 different levels of strontium enriched ASW and the strontium peak profile across this image. The four Sr bands and the four peaks in the plot correspond to 1, 0.5, 0.25, and 0.1 mM Sr enriched ASW, from left to right.
FIG. 3. Linear relationship between level of Sr in the ASW and the Sr:Ca ratio from the shells of three different barnacles.